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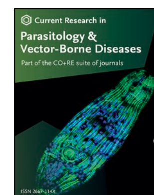

Development, validation, and pilot application of a high throughput molecular xenomonitoring assay to detect *Schistosoma mansoni* and other trematode species within *Biomphalaria* freshwater snail hosts

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ABSTRACT

Schistosomiasis is a neglected tropical disease (NTD) caused by infection with parasitic trematodes of the genus *Schistosoma* that can lead to debilitating morbidity and mortality. The World Health Organization recommend molecular xenomonitoring of *Biomphalaria* spp. freshwater snail intermediate hosts of *Schistosoma mansoni* to identify highly focal intestinal schistosomiasis transmission sites and monitor disease transmission, particularly in low-endemicity areas. A standardised protocol to do this, however, is needed. Here, two previously published primer sets were selected to develop and validate a multiplex molecular xenomonitoring end-point PCR assay capable of detecting *S. mansoni* infections within individual *Biomphalaria* spp. missed by cercarial shedding. The assay proved highly sensitive and highly specific in detecting and amplifying *S. mansoni* DNA and also proved highly sensitive in detecting and amplifying non-*S. mansoni* trematode DNA. The optimised assay was then used to screen *Biomphalaria* spp. collected from a *S. mansoni*-endemic area for infection and successfully detected *S. mansoni* infections missed by cercarial shedding as well as infections with non-*S. mansoni* trematodes. The continued development and use of molecular xenomonitoring assays such as this will aid in improving disease control efforts, significantly reducing disease-related morbidities experienced by those in schistosomiasis-endemic areas.

1. Introduction

Schistosomiasis is a neglected tropical disease (NTD) caused by infection with parasitic trematodes of the genus *Schistosoma* that can lead to debilitating morbidity and mortality (Colley et al., 2014). Whilst it is estimated that over 230 million people are currently infected globally, over 90% of all cases occur within sub-Saharan Africa (McManus et al., 2018). Of these, around one-third are deemed intestinal schistosomiasis, caused predominantly by infection with *Schistosoma mansoni* but also less commonly by infection with *Schistosoma intercalatum* and *Schistosoma guineensis* in some restricted areas of central Africa (Tchuem Tchuenté et al., 2017). *Schistosoma mansoni* is transmitted *via* obligate freshwater snail intermediate hosts of the genus *Biomphalaria* (Gastropoda: Planorbidae) (Morgan et al., 2001). Human infection occurs through contact with contaminated bodies of freshwater, where infectious *S. mansoni* larval stages (cercariae) shed from

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infected *Biomphalaria* spp. hosts contact their definitive human host and penetrate the skin. As such, intestinal schistosomiasis is often highly prevalent in rural areas lacking adequate water, sanitation, and hygiene (WASH) infrastructure, where entire communities rely on bodies of freshwater as a source of drinking water, food (*via* fishing), a place to bathe, a place to tend livestock, and for recreation (Grimes et al., 2015).

Intestinal schistosomiasis is typically diagnosed using Kato-Katz faecal-egg microscopy (Katz et al., 1972; WHO, 2022). Whilst this method is relatively inexpensive and can be carried out at the point-of-care, it is considered low-throughput and lacks sensitivity, particularly when attempting to diagnose individuals harbouring low-intensity infections (Utzinger et al., 2015). Reliably monitoring disease transmission in areas of low endemicity, such as those that have undergone repeated annual or bi-annual mass drug administration (MDA) with the anthelmintic drug praziquantel (Knopp et al., 2011) or in areas where a recent outbreak of infections has occurred (Kayuni et al., 2020), can therefore be extremely difficult using this method alone. Because of these difficulties, detecting and monitoring the transmission of intestinal schistosomiasis is often also carried out through malacological surveillance of *Biomphalaria* spp. freshwater snail intermediate hosts followed by cercarial shedding analysis to identify sites of active transmission. This method, however, is also extremely insensitive, primarily because not all freshwater snails harbouring mature, or patent, infections will be actively shedding cercariae and because freshwater snails harbouring prepatent infections will also not shed cercariae (Tavalire et al., 2016; Weerakoon et al., 2018; Kamel et al., 2021). In addition, cercarial shedding can often be misleading owing to morphological similarities between human-infecting and non-human-infecting trematode cercariae. For example, *S. mansoni* and *Schistosoma rodhaini* (a morphologically indistinguishable rodent-infecting sister species of *S. mansoni*) are both transmitted by *Biomphalaria* spp., and so molecular methods of differentiating these species are needed (Norton et al., 2008; Lu et al., 2016).

An alternative approach used to detect vector-borne pathogens within their host vectors is through molecular xenomonitoring. This method involves the detection and amplification of pathogen DNA from within host vectors typically through some variety of polymerase chain reaction (PCR) (Lu et al., 2016; Schols et al., 2019; Pennance et al., 2020), but also through more novel isothermal nucleic acid amplification technologies such as loop-mediated isothermal amplification (LAMP) (García-Bernalt Diego et al., 2021) or recombinase polymerase/aided amplification (RPA/RAA) (Mesquita et al., 2022). Molecular xenomonitoring has been successfully used to detect and monitor the transmission of several vector-borne parasitic diseases, including malaria (Cameron and Ramesh, 2021), lymphatic filariasis and onchocerciasis (Pilotte et al., 2017), trypanosomiasis (Grébaut et al., 2016), fascioliasis (Rathinasamy et al., 2018), urogenital schistosomiasis (Schols et al., 2019; Pennance et al., 2020) and intestinal schistosomiasis (Lu et al., 2016; Andrus et al., 2023). As such, the World Health Organisation (WHO) has recommended the continued development and implementation of molecular xenomonitoring approaches to identify highly focal sites of ongoing schistosomiasis transmission, particularly in low-transmission areas nearing disease elimination (WHO, 2022). In doing so, more impactful disease control measures can be implemented, such as ceasing costly MDA programmes in areas that would provide no health benefit and concentrating resources in areas where ongoing transmission has been identified.

However, molecular xenomonitoring approaches can be expensive and require sophisticated laboratory infrastructure as well as specialised personnel. In addition, as so few (usually $< 5\%$) of collected freshwater snails are typically found to be harbouring *Schistosoma* spp. infections when using molecular xenomonitoring approaches, many freshwater snails (ideally > 50 from each malacological surveillance site) is required to thoroughly assess any given location for evidence of schistosomiasis transmission. When developing molecular xenomonitoring methods, the overall assay cost, ability to be implemented in endemic areas, throughput, ease-of-use, speed, reliability, and ease of interpretation should therefore be carefully considered (Kamel et al., 2021). This includes consideration towards the essential preliminary steps needed to isolate DNA from freshwater snail tissues (Adema, 2021).

Here, we aimed to develop a high-throughput, easily interpreted, and reliable molecular xenomonitoring assay for the detection of *S. mansoni* and other trematode species within *Biomphalaria* spp. freshwater snail hosts. Whilst previous molecular xenomonitoring assays have been used to detect *S. mansoni* within *Biomphalaria* spp. hosts (Lu et al., 2016), these assays do not detect other species of trematode which may influence *S. mansoni* development within *Biomphalaria* spp., therefore impacting disease transmission, nor do they use an internal DNA extraction/PCR control, meaning a negative result may be interpreted as non-infection rather than DNA extraction or PCR reaction failure (Pennance et al., 2020). We therefore opted for a multiplex PCR approach capable of also detecting non-*S. mansoni* trematodes whilst also incorporating an internal reaction control (a *Biomphalaria* DNA locus). In addition, we used a rapid, automated, and high-throughput DNA extraction methodology together with a high-throughput and easily interpreted end-point PCR approach.

2. Materials and methods

2.1. *Biomphalaria spp. freshwater snails and Schistosoma spp. trematodes used during assay development, validation, and pilot application*

Biomphalaria spp. freshwater snails and *Schistosoma* spp. trematodes (adult worms, miracidia, and ova) used during assay development and validation were provided by the Schistosome and Snail Resource (SSR) (SSR, 2024), the Schistosomiasis Collection at the Natural History Museum (SCAN) archive (Emery et al., 2012) and the Biomedical Research Institute (BRI) (BRI, 2024a). In addition, 92 *Biomphalaria* spp. snails used during assay pilot application had been previously collected from a single malacological surveillance site situated on the southern shoreline of Lake Malawi, Mangochi District, Malawi (an area endemic for *S. mansoni* transmission; Kayuni et al., 2020) as part of the *Hybridisations in UroGenital Schistosomiasis* (HUGS) project (Archer et al., unpublished). Details of all *Biomphalaria* and *Schistosoma* material including sample IDs, collection location or laboratory strain, and DNA extraction method are outlined in Table 1.

2.2. *Assay development and validation*

2.2.1. *Target DNA loci and primer selection*

Two previously published primer sets were selected for development of the multiplex molecular xenomonitoring end-point PCR assay. The commonly used ETTS2 forward and ETTS1 reverse primers were selected to target and amplify the complete nuclear internal transcribed spacer (ITS) region of members of both Gastrapoda (~1250 bp) and Trematoda (~1005 bp) (Kane and Rollinson, 1994). This primer set has been used previously within a multiplex molecular xenomonitoring end-point PCR assay to detect trematode infections (including *Schistosoma* spp.) within *Bulinus* spp. freshwater snail hosts (Pennance et al., 2020). In addition, *S. mansoni* NADH dehydrogenase subunit 5 (ND5) forward and reverse primers were selected to target and amplify a 305-bp region of the *S. mansoni* mitochondrial ND5 gene (Lu et al., 2016). Details of all primer oligonucleotide sequences are provided in Table 2.

2.2.2. *Specificity testing and multiplex assay optimisation*

2.2.2.1. Singleplex Biomphalaria ITS and Schistosoma ITS PCR testing. To confirm amplification of the target Gastrapoda (*Biomphalaria*) ITS and Trematoda (*Schistosoma*) ITS loci, singleplex PCR reactions were performed using ETTS2 and ETTS1 primers and *B. pfeifferi* (*Bp*), *B. sudanica*

Table 1
Biomphalaria and *Schistosoma* material used during development, validation, and pilot application of the molecular xenomonitoring end-point PCR assay.

Sample ID	Specimen	Collection location or laboratory strain	*S. mansoni* infection status	DNA extraction method	Provided by
Bp	*B. pfeifferi* ($n = 1$)	Collected: Lake Victoria, Uganda	Non-infected	DNeasy blood and tissue kit (Qiagen, UK) using standard protocol (Pennance et al., 2018) with minor modifications[a]	SSR
Bs	*B. sudanica* ($n = 1$)	Collected: Lake Victoria, Tanzania	Non-infected		SCAN
Bc	*B. choanomphala* ($n = 1$)	Collected: Lake Victoria, Tanzania	Non-infected		SCAN
Sm	*S. mansoni* adult worms ($n = 3$, collectively extracted)	Laboratory passaged: Puerto Rico strain	Not applicable	DNeasy blood and tissue kit (Qiagen, UK) using standard protocol (Webster et al., 2012)	SSR
Sr	*S. rodhaini* adult worms ($n = 3$, collectively extracted)	Laboratory passaged: Uganda strain	Not applicable		SSR
Sh	*S. haematobium* adult worms ($n = 3$, collectively extracted)	Laboratory passaged: Senegal strain	Not applicable		SCAN
Sm-mir(1)	*S. mansoni* miracidia ($n = 6$, individually extracted)	Laboratory passaged: Puerto Rico strain	Not applicable	DNeasy blood and tissue kit (Qiagen, UK) using standard protocol (Webster et al., 2012) with minor modifications[b]	BRI
Bg+*Sm*(1)	*B. glabrata* exposed to one *S. mansoni* miracidium ($n = 5$, individually extracted)	Laboratory-bred: Brazil (BB02) strain, originally collected in 2002	Infected: Control laboratory infection with one *S. mansoni* miracidium (Puerto Rico strain) (*Section 2.2.4.1*)	DNeasy blood and tissue kit (Qiagen, UK), 24 h post miracidia exposure using standard protocol (Pennance et al., 2018) with minor modifications[a]	*B. glabrata* provided by SSR; *S. mansoni* miracidia provided by BRI
Bg+*Sm*(5)	*B. glabrata* exposed to five *S. mansoni* miracidia ($n = 5$, individually extracted)		Infected: Control laboratory infection with 5 *S. mansoni* miracidia (Puerto Rico strain) (*Section 2.2.4.1*)		
Bg	*B. glabrata* ($n = 2$ individually extracted)		Non-infected (*Section 2.2.4.1*)		SSR
*Biomph*91 (mal)	*Biomphalaria* spp. ($n = 91$, individually extracted)	Collected: Lake Malawi, Malawi (*Section 2.3.1*)	Unknown: Not shedding *S. mansoni* cercariae	BioSprint 96 workstation and BioSprint 96 DNA Blood Kit (Qiagen, UK) using standard protocol (Pennance et al., 2020)	Not applicable
Bp+*Sm* (mal)	*B. pfeifferi* infected with *S. mansoni* ($n = 1$)	Collected: Lake Malawi, Malawi (*Section 2.3.1*)	Infected: Natural infection, shedding *S. mansoni* cercariae		Not applicable

Abbreviations: SSR, Schistosome and Snail Resource, London, UK (SSR, 2024); SCAN, Schistosome Collections at Natural History Museum, London, UK (Emery et al., 2012); BRI, Biomedical Research Institute, Maryland, USA.
[a] Double volume cell lysis buffers used.
[b] Half volume cell lysis buffers and elution buffer used.

Table 2
Primer sequences used during development, validation, and pilot application of the molecular xenomonitoring end-point PCR assay.

Name	Target (amplicon length)	Oligonucleotide sequence (5′–3′)	Reference
ETTS2 (F)	*Biomphalaria* ITS (~1250 bp) & *Schistosoma* ITS (~1005 bp)	TGCTTAAGTTCAGCGGG	Kane and Rollinson (1994); Pennance et al. (2020)
ETTS1 (R)		TAACAAGGTTTCCGTAGGTGA	
ND52 (F)	*S. mansoni* ND5 (305 bp)	ATTAGAGGCAATGCGTGCTC	Lu et al. (2016)
ND52 (R)		ATTGAACCAACCCCAAATCA	

Abbreviations: F, forward primer; R, reverse primer.

(*Bs*), *B. choanomphala* (*Bc*), *S. mansoni* (*Sm*), *S. rodhaini* (*Sr*), *S. haematobium* (*Sh*) and *B. pfeifferi* infected with *S. mansoni* (*Bp*+*Sm* (mal)) sample DNA. These were carried out using 25 μl PCR reactions made up of 2 μl template DNA (2 ng/μl) from each DNA sample, 10 pmol of each primer, 21 μl ddH_2O and one Illustra PuReTaq ready-to-go PCR bead (Sigma-Aldrich, Dorset, UK). One no-template negative control using 2 μl ddH_2O in place of DNA was also used. Singleplex PCR reactions were performed using the following cycling conditions: 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 58 °C and 90 s at 72 °C; and 10 min at 72 °C. This was then repeated using an annealing temperature of 60 °C (rather than 58 °C) to check for improved target detection and amplification. Amplicons were visualised by running 7.5 μl PCR product mixed with 2 μl 5× loading buffer blue (Bioline, Essex, UK) stained with GelRed on a 2% agarose gel.

PCR products generated using *Bp*, *Bs*, *Bc*, *Sm*, *Sr* and *Sh* sample DNA were purified using the QIAquick PCR purification kit (Qiagen, Manchester, UK) according to the manufacturer's instructions and Sanger sequenced in the forward direction using a dilution of the ETTS2 forward primer. PCR products generated using *Bp*+*Sm*(mal) sample DNA were visualised under UV light and both ~1250-bp *Biomphalaria* and ~1005-bp *Schistosoma* bands were individually excised using a fresh scalpel. Excised gel bands were individually purified using the QIAquick Gel purification kit (Qiagen) according to the manufacturer's instructions and were then purified as described above prior to Sanger sequencing in the forward direction again using a dilution of the ETTS2 forward primer. Sequence data were visualised, trimmed, and edited using Geneious Prime version 2023.01 (Biomatters, LTD) and identified using the Basic Local Alignment Search Tool (BLAST) algorithm within the NCBI database (Geer et al., 2009).

2.2.2.2. Singleplex S. mansoni ND5 PCR testing. To confirm amplification of the target *S. mansoni* ND5 locus, the above singleplex PCRs were repeated using the *S. mansoni* ND5 forward and reverse primers in place of ETTS2 and ETTS1 primers. PCRs were again initially performed using an annealing temperature of 58 °C and then repeated using an annealing temperature of 60 °C to check for improved target detection and amplification. Amplicons were visualised as described above. All *S. mansoni* ND5 PCR products were then purified as described above, and Sanger sequenced in the forward direction using a dilution of the ND52 forward primer. Sequence data were visualised, trimmed, edited, and

identified as described above.

2.2.2.3. Multiplex Biomphalaria ITS, Schistosoma ITS and S. mansoni ND5 PCR testing. Multiplex PCR reactions were then performed incorporating both ITS and *S. mansoni* ND5 primer sets using *Bp*, *Sm*, *Sr*, *Sh* and *Bp*+*Sm*(mal) sample DNA. *Bp*+*Sm*(mal) reactions were performed in triplicate. These were carried out using 25 μl PCR reactions made up of 2 μl template DNA (2 ng/μl) from each DNA sample, 10 pmol of each primer, 19 μl ddH_2O and one Illustra PuReTaq ready-to-go PCR bead (Sigma-Aldrich). One no-template negative control using 2 μl ddH_2O in place of DNA was also used. Multiplex PCR reactions were again initially performed using the following cycling conditions: 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 58 °C and 90 s at 72 °C; and 10 min at 72 °C, and then repeated using an annealing temperature of 60 °C (rather than 58 °C) to check for improved target detection and amplification. Amplicons were visualised as described above.

2.2.3. Multiplex assay sensitivity testing

2.2.3.1. Multiplex assay using a single S. mansoni miracidium. The multiplex PCR was performed as described above (annealing temperature 60 °C) using 2 μl *S. mansoni* miracidia (*Sm*-mir(1)) template DNA (2 ng/μl) extracted from one single *S. mansoni* miracidium provided by the BRI. This was carried out using all six individual *Sm*-mir(1) samples. The PCR assay included one positive control using 2 μl *Sm* sample DNA (2 ng/μl) and one no-template negative control using 2 μl ddH_2O in place of DNA. Amplicons were visualised as described above.

2.2.3.2. Analytical limit-of-detection testing. *Sm* and *Sr* sample DNA was normalised to a concentration of 2 ng/μl and then serially diluted to 0.2 ng/μl, 0.02 ng/μl, and 2 pg/μl using ddH_2O. Multiplex PCRs were then performed as described above (annealing temperature 60 °C) using 1 μl of each DNA concentration together with 1 μl *Bp* sample DNA (2 ng/μl). All reactions were performed in triplicate. The PCR assay included four positive controls: two using 2 μl *Bp* sample DNA (2 ng/μl), one using 2 μl *Sm* sample DNA (2 ng/μl) and one using 2 μl *Sr* sample DNA (2 ng/μl). Two no-template negative controls using 2 μl ddH_2O in place of DNA were also used. Amplicons were visualised as described above.

2.2.4. Multiplex assay validation

2.2.4.1. Controlled laboratory infection of B. glabrata with S. mansoni miracidia. Twelve laboratory-bred *B. glabrata* freshwater snails (all ~3 months old with a discoid shell height of ~8 mm) were individually placed within plastic pots containing 30 ml ddH_2O. A single *S. mansoni* miracidium was then pipetted into five of the pots. Five *S. mansoni* miracidia were then pipetted into a further five pots. Miracidia were not added to the two remaining pots. All twelve pots were then covered to ensure snail submersion and stored at 27 °C for 24 h, after which DNA was isolated from all *B. glabrata* whole tissues. All pots were examined under dissecting microscope to check for the presence or absence of miracidia immediately after *B. glabrata* were removed.

2.2.4.2. Molecular xenomonitoring PCR. The multiplex molecular xenomonitoring PCR was performed as described above (annealing temperature 60 °C) using all 12 *B. glabrata* DNA isolates. The PCR assay included two positive controls using 2 μl *Bp* sample DNA (2 ng/μl) and 2 μl *Sm* sample DNA (2 ng/μl). One no-template negative control using 2 μl ddH_2O in place of DNA was also used. Amplicons were visualised as described above.

2.3. Assay pilot application

2.3.1. Collection and cercarial shedding of Biomphalaria from a S. mansoni-endemic area

Biomphalaria spp. previously collected from a single malacological surveillance site (October 2022) situated on the southern shoreline of Lake Malawi, Mangochi District, Malawi (an area endemic for *S. mansoni* transmission) were used during assay pilot application. All freshwater snails were collected according to a standard protocol and screened for patent *Schistosoma* spp. infection by cercarial shedding also according to a standard protocol (Pennance et al., 2022). All necessary personal protective equipment needed to prevent skin contact with contaminated freshwater during malacological collections and cercarial shedding was used at all times.

One of the collected specimens of *Biomphalaria* (0.7%) was found to be actively shedding *Schistosoma* spp. cercariae, and so this specimen was individually preserved fully submerged in 100% ethanol within a 2 ml screwcap tube. All remaining specimens of *Biomphalaria* were collectively preserved fully submerged in 100% ethanol within three 20 ml glass screwcap tubes and transported to the Natural History Museum (NHM), London, UK, under ambient conditions for DNA extraction (June 2023) and molecular analyses. DNA was isolated from each ethanol-preserved specimen of *Biomphalaria* in batches of 92 samples using the BioSprint 96 workstation and BioSprint 96 DNA Blood Kit (Qiagen) according to a previously outlined protocol (Pennance et al., 2020). This magnetic bead-based DNA extraction protocol was chosen as it allows for rapid and high-throughput multiple-sample processing using a 96-well plate format.

The shedding specimen of *Biomphalaria* was confirmed as *B. pfeifferi* through genotyping of the mitochondrial *cox*1 region according to a standard protocol (Standley et al., 2011; GenBank: OR880348) and the *Schistosoma* spp. cercariae shed from this specimen were confirmed as *S. mansoni* through genotyping of the mitochondrial *cox*1 and nuclear ITS DNA regions also according to a standard protocol (Webster et al., 2013; GenBank: PP390203 and PP388909, respectively).

2.3.2. Molecular xenomonitoring PCR

The multiplex molecular xenomonitoring PCR was used to screen a subset of the collected *Biomphalaria* DNA isolates for infection with *S. mansoni* and other trematodes. This subset was comprised of 92 *Biomphalaria* DNA isolates contained within a single 96-well plate that included the one *Biomphalaria* (*B. pfeifferi*) actively shedding *S. mansoni* cercariae (*Bp*+*Sm*(mal) sample DNA; Table 1) as well as 91 non-shedding *Biomphalaria* (*Biomph*91(mal) DNA samples; Table 1). The PCR was performed as described above (annealing temperature 60 °C) and included three positive controls using 2 μl *Bp* sample DNA (2 ng/μl), 2 μl *Sm* sample DNA (2 ng/μl), and 2 μl *Sr* sample DNA (2 ng/μl). One no-template negative control using 2 μl ddH_2O in place of DNA was also used. Amplicons were visualised as described above.

Samples that amplified only the *Biomphalaria* ITS locus (considered an internal PCR control) were considered *Biomphalaria* negative for trematode infection of any sort, including *S. mansoni*. Samples that successfully amplified all three target loci were considered *Biomphalaria* positive for *S. mansoni* infection, as well as potentially other species of the Trematoda. Samples that amplified only the *Biomphalaria* and Trematoda ITS loci were considered *Biomphalaria* positive for trematode infection, excluding *S. mansoni*. Samples that failed to amplify the *Biomphalaria* ITS locus (regardless of Trematoda ITS and *S. mansoni* ND5 amplification outcome), were considered to have failed PCR and were repeated. Samples that failed to amplify this locus during the repeat screen were considered to have failed DNA extraction and were omitted from any further analysis.

2.3.3. Confirmatory S. mansoni ND5 PCR and genotyping

To confirm infection with *S. mansoni* and to validate our protocol, all samples that successfully amplified all three target loci were subjected to an additional singleplex PCR to again amplify the *S. mansoni* ND5 locus, as described above (*Section 2.2.2.2*; annealing temperature of 60 °C). Amplicons were visualised, purified, Sanger sequenced and identified as described above (*Section 2.2.2.2*).

2.3.4. Conformation and identification of Biomphalaria infections with other trematode species

To confirm infection with and identify non-*S. mansoni* trematode species infections, and to validate our protocol, all samples that successfully amplified both *Biomphalaria* and Trematoda ITS loci only were subjected to an additional singleplex PCR to again amplify the *Biomphalaria* ITS and Trematoda ITS loci, as described above (*Section 2.2.2.1*; annealing temperature of 60 °C). Agarose gel bands were then visualised under UV light and the ~1005-bp Trematoda gel band was excised using a fresh scalpel. Excised gel bands were purified using the QIAquick Gel purification kit (Qiagen) according to the manufacturer's instructions and were then purified as described above prior to Sanger sequencing in the forward direction (*Section 2.2.2.1*). Sequence data were visualised, trimmed, edited, and identified as described above (*Section 2.2.2.1*).

2.4. Ethical considerations

Live *B. glabrata* (Brazil BB02 strain; routinely maintained according to previously outlined standard operating procedures (BRI, 2024b) were provided by the Schistosome and Snail Resource (SSR, 2024) based across both the Natural History Museum (NHM), London, UK, and the London School of Hygiene and Tropical Medicine (LSHTM), London, UK (Wellcome Trust Biomedical Resource Grant 221368/Z/20/Z (2021–2026)). Controlled laboratory infection of *B. glabrata* with *S. mansoni* was performed at the NHM using *S. mansoni* (Puerto Rico strain) miracidia hatched from ova provided by the Biomedical Research Institute (BRI, 2024a), Rockville, Maryland, USA (*Section 2.2.4.1*). Ova were isolated and collected from the liver of infected mice following percutaneous tail exposure to *S. mansoni* cercariae (BRI, 2024a). Animal use was approved the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute for the Animal Use Protocol, #18-01. Ethical approval and research authorisations for the collection of *Biomphalaria* spp. from the southern shoreline of Lake Malawi, Malawi, were approved in the UK by the Liverpool School of Tropical Medicine (LSTM) Research Ethics Committee (application 17-018) and in Malawi by the National Health Sciences Research Committee (1805).

3. Results

3.1. Assay development

3.1.1. Specificity testing and multiplex assay optimisation

3.1.1.1. Singleplex Biomphalaria ITS and Schistosoma ITS PCR testing. All *Biomphalaria* ITS and *Schistosoma* ITS singleplex reactions performed as anticipated with no non-target amplification. A single ~1250-bp amplicon was produced when using *Bp*, *Bs* and *Bc* sample DNA, a single ~1005-bp amplicon was produced when using *Sm*, *Sr* and *Sh* sample DNA, and two amplicons were produced when using *Bp*+*Sm*(mal) sample DNA (~1250 bp and ~1005 bp in length). Increasing the PCR annealing temperature to 60 °C did not appear to have any observable effect on PCR performance when compared to 58 °C. All *Biomphalaria* and *Schistosoma* ITS amplicons (using 60 °C annealing temperature) were successfully confirmed using Sanger sequencing.

3.1.1.2. Singleplex S. mansoni ND5 PCR testing. All *S. mansoni* ND5 singleplex reactions also performed as anticipated with no non-target amplification. A single 305-bp amplicon was produced when using *Sm* sample DNA and when using *Bp*+*Sm*(mal) sample DNA. No amplicons were produced when using *Bp*, *Bs*, *Bc*, *Sr* and *Sh* sample DNA. When using an annealing temperature of 58 °C, the 305-bp *S. mansoni* ND5 agarose gel bands were present but faint. By comparison, when increasing the annealing temperature to 60 °C, these agarose gel bands were much stronger. Both *Sm* and *Bp*+*Sm*(mal) ND5 amplicons (using an annealing temperature of 60 °C) were successfully confirmed using Sanger sequencing.

3.1.1.3. Multiplex Biomphalaria ITS, Schistosoma ITS and S. mansoni ND5 PCR testing. All multiplex reactions performed as anticipated with no non-target amplification. A single ~1250-bp amplicon was produced when using *Bp* sample DNA. Two amplicons were produced when using *Sm* sample DNA (~1005-bp and 305-bp in length). A single ~1005-bp amplicon was produced when using *Sr* and *Sh* sample DNA. All three target amplicons were produced when using *Bp*+*Sm*(mal) sample DNA in all three replicates (~1250 bp, ~1005 bp and 305 bp in length; Fig. 1). Increasing the annealing temperature to 60 °C again improved the strength of the 305-bp *S. mansoni* ND5 agarose gel band in all reactions where *S. mansoni* DNA was present when compared to an annealing temperature of 58 °C, whilst having no observable effect on *Biomphalaria* ITS or *Schistosoma* ITS amplicons. When using both annealing temperatures, the *Schistosoma* ITS gel band was less strong in multiplex reactions using *Bp*+*Sm*(mal) sample DNA when compared to *Biomphalaria* ITS and *Schistosoma* ITS singleplex reactions using the same DNA and when using *Sm*, *Sr* and *Sh* sample DNA in multiplex reactions.

3.1.2. Multiplex assay sensitivity testing

Both ~1005-bp *Schistosoma* ITS and 305-bp *S. mansoni* ND5 amplicons were produced when using a single *S. mansoni* miracidium in all six *Sm*-mir(1) DNA samples. Furthermore, the multiplex assay proved capable of detecting *S. mansoni* DNA with a limit-of-detection of between 0.2 ng and 0.02 ng (Fig. 2). Whilst the strength of the

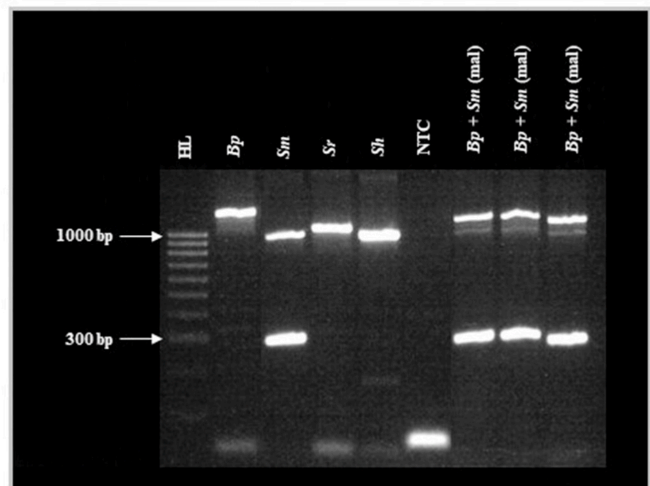

Fig. 1. Agarose gel image of the multiplex molecular xenomonitoring PCR performed using an annealing temperature of 60 °C. *Abbreviations*: HL, HyperLadder 100 bp (BioLine, UK); *Bp*, *Biomphalaria pfeifferi* sample DNA; *Sm*, *Schistosoma mansoni* sample DNA; *Sr*, *S. rodhaini* sample DNA; *Sh*, *S. haematobium* sample DNA; NTC, no template ddH$_2$O negative control; *Bp*+*Sm* (mal): *B. pfeifferi* infected with *S. mansoni* (collected in Malawi) sample DNA. All *Biomphalaria* ITS, *Schistosoma* ITS and *S. mansoni* ND5 multiplex reactions performed as anticipated with no non-target amplification. GelRed loading buffer stain may affect PCR amplicon migration.

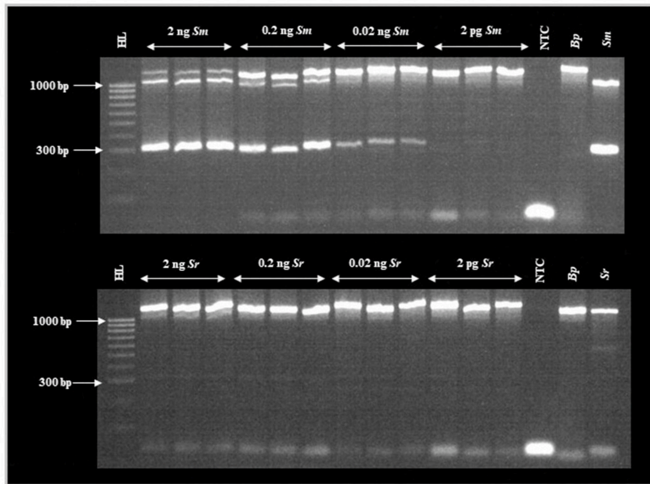

Fig. 2. Agarose gel image of the multiplex molecular xenomonitoring PCR during sensitivity (analytical limit-of-detection) testing. *Abbreviations*: HL, HyperLadder 100 bp (BioLine, UK); *Sm*, *Schistosoma mansoni* sample DNA; *Sr*, *S. rodhaini* sample DNA; NTC, no template ddH_2O negative control; *Bp*, *Biomphalaria pfeifferi* sample DNA. Whilst the strength of the *Biomphalaria* ITS agarose gel band remained constant in all reactions, as *S. mansoni* and *S. rodhaini* DNA concentration decreased, the strength of the *Schistosoma* ITS gel bands (both *S. mansoni* and *S. rodhaini*) and the *S. mansoni* ND5 gel band decreased. The multiplex assay proved capable of detecting *S. mansoni* DNA with a limit-of-detection of between 0.2 ng and 0.02 ng and proved capable of detecting *S. rodhaini* DNA with a limit-of-detection of between 2 ng and 0.2 ng. GelRed loading buffer stain may affect PCR amplicon migration.

Biomphalaria ITS agarose gel band remained constant in all reactions, the strength of the *Schistosoma* ITS gel band and the *S. mansoni* ND5 gel band decreased as *S. mansoni* DNA concentration decreased. The multiplex assay also proved capable of detecting *S. rodhaini* DNA with a limit-of-detection of between 2 ng and 0.2 ng (Fig. 2). Again, whilst the strength of the *Biomphalaria* ITS agarose gel band remained constant in all reactions, the strength of the *Schistosoma* ITS gel band decreased as *S. rodhaini* DNA concentration decreased. All control reactions performed as anticipated.

3.1.3. Multiplex assay validation

Upon microscopic examination, no miracidia appeared present in any pots immediately after removal of *B. glabrata*. The multiplex assay proved capable of detecting *S. mansoni* DNA within *Biomphalaria* exposed to a single miracidium 24 h post-exposure (Fig. 3). The *S. mansoni* ND5 locus was detected and visibly amplified (albeit, faintly) in four out of five *B. glabrata* exposed to a single miracidium. However, the *Schistosoma* ITS locus was not visibly amplified in any of these samples. The *S. mansoni* ND5 locus was detected and amplified in all five *B. glabrata* exposed to five miracidia 24 h post-exposure and the strength of these gel bands appeared stronger than those generated after exposure to a single miracidium 24 h post-exposure (Fig. 3). However, the *Schistosoma* ITS locus was detected and visibly amplified (albeit, faintly) in only three of these five samples. The *Biomphalaria* ITS locus was amplified in all 12 *B. glabrata* samples. All control reactions performed as anticipated.

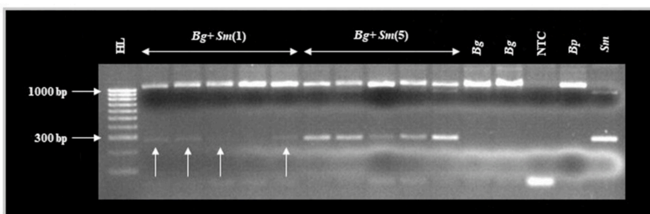

Fig. 3. Agarose gel image of the multiplex molecular xenomonitoring PCR during assay validation (controlled laboratory infection of *B. glabrata* with *S. mansoni* miracidia). *Abbreviations*: HL, HyperLadder 100 bp (BioLine, UK); *Bg*+*Sm*(1), sample DNA isolated from individual *Biomphalaria glabrata* exposed to a single miracidium of *Schistosoma mansoni*; *Bg*+*Sm*(5), sample DNA isolated from individual *B. glabrata* exposed to five miracidia of *S. mansoni*; *Bg*, *B. glabrata* sample DNA isolated from individual *B. glabrata* not exposed to miracidia of *S. mansoni*; NTC, no template ddH_2O negative control; *Bp*, *B. pfeifferi* sample DNA; *Sm*, *S. mansoni* sample DNA. *Schistosoma mansoni* DNA was detected in four of all five *Bg*+*Sm*(1) samples (highlighted by upward-facing white arrows). *Schistosoma mansoni* DNA was also detected in all five *Bg*+*Sm*(5) samples. GelRed loading buffer stain may affect PCR amplicon migration.

3.2. Assay pilot application

The *Biomphalaria* ITS locus was amplified in all *Biomph*91(mal) DNA samples as well as in the *Bp*+*Sm*(mal) DNA sample, and so DNA extraction and all PCR reactions were deemed successful (Fig. 4). All three target loci were amplified in the *Bg*+*Sm*(mal) sample DNA, as well as two *Biomph*91(mal) DNA samples, indicating infection with *S. mansoni* in three of all 92 (3.3%) specimens of *Biomphalaria*. In one of these *Biompha*91(mal) samples, the *Schistosoma* ITS locus was amplified only faintly (Fig. 4, sample B). In addition, both the *Biomphalaria* ITS and Trematoda ITS loci only were amplified in two *Biomph*91(mal) DNA samples, indicating infection with non-*S. mansoni* trematodes in two of all 92 (2.2%) specimens of *Biomphalaria* (Fig. 4). All control reactions performed as anticipated.

Infection with *S. mansoni* was confirmed in all three *S. mansoni*-infected samples through singleplex PCR, Sanger sequencing and BLAST analysis of the *S. mansoni* ND5 locus, validating our molecular xenomonitoring protocol. The prevalence of *S. mansoni* infection in these 92 *Biomphalaria* freshwater snails was therefore increased from 1.1% to 3.3% through use of the multiplex molecular xenomonitoring PCR. Both trematode infections were confirmed as *Uvulifer* spp. (closest match GenBank: MK604882; mean percent identity score: 96.6; query cover

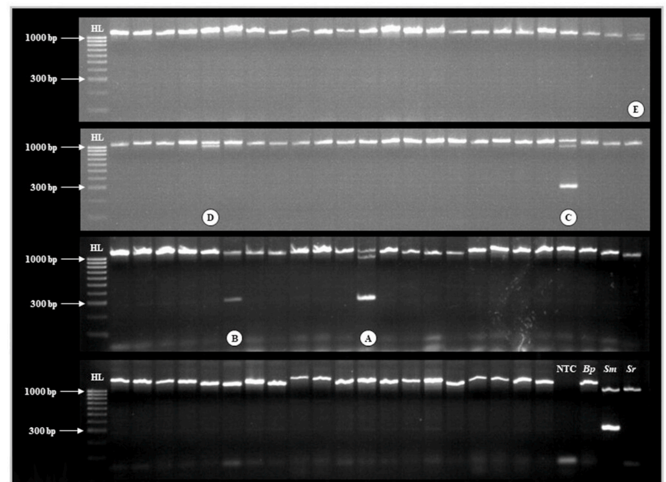

Fig. 4. Agarose gel image of the high throughput multiplex molecular xenomonitoring PCR assay during pilot application. *Abbreviations*: HL, HyperLadder 100 bp (BioLine, UK); NTC, no template ddH_2O negative control; *Bp*, *Biomphalaria pfeifferi* sample DNA; *Sm*, *Schistosoma mansoni* sample DNA; *Sr*, *S. rodhaini* sample DNA. **A**: *Bp*+*Sm*(mal) sample infected with *S. mansoni* (detected during cercarial shedding); **B** and **C**: Two *Biomph*91(mal) samples infected with *S. mansoni* (not detected during cercarial shedding); **D** and **E**: Two *Biomph*91(mal) samples infected with *Uvulifer* spp. All remaining samples are *Biomph*91(mal) samples not found to be infected with any trematode species, including *S. mansoni*. GelRed loading buffer stain may affect PCR amplicon migration.

86.3%) through agarose gel excision, purification, Sanger sequencing and BLAST analysis of the Trematoda ITS locus. This diplostomid trematode has a three-host life-cycle: specific genera of freshwater snails including *Biomphalaria* (López-Hernández et al., 2019), freshwater sunfish, and piscivorous birds such as kingfishers (highly prevalent along this shoreline of Lake Malawi; Johnston, 1989).

4. Discussion

In areas of low disease endemicity, highly sensitive and reliable methods of detecting *S. mansoni* transmission are crucial for successful disease monitoring and control. Molecular xenomonitoring is a valuable and extremely sensitive approach that can be used to detect and monitor the transmission of vector-borne pathogens within their host vectors. Here, we aimed to develop a high throughput, easily interpreted, and reliable molecular xenomonitoring assay for the detection of *S. mansoni* and other trematode species within *Biomphalaria* spp. freshwater snail hosts.

The molecular xenomonitoring assay was successful in detecting and amplifying the ITS loci of a range of *Biomphalaria* and *Schistosoma* species. The *Biomphalaria* ITS locus was therefore deemed suitable as an internal DNA extraction/PCR reaction control and the ETTS2 forward and ETTS1 reverse primers were deemed capable of detecting trematode species other than *S. mansoni*, as also shown previously using non-*Schistosoma* trematode infections in *Bulinus* spp. freshwater snails (Pennance et al., 2020). In addition, the assay proved highly specific in detecting and amplifying the *S. mansoni* ND5 locus in both singleplex and multiplex reactions. Of note, the *S. mansoni*-specific ND5 primers did not cross-react with sister species *S. rodhaini* adult worm DNA at any point during these analyses.

The molecular xenomonitoring assay also proved highly sensitive during sensitivity testing and during controlled laboratory infection of *B. glabrata* with *S. mansoni* miracidia testing. Both ~1005-bp *Schistosoma* ITS and 305-bp *S. mansoni* ND5 amplicons were successfully produced when using a single *S. mansoni* miracidium, and during analytical limit-of-detection testing, the assay was able to detect extremely low concentrations of *S. mansoni* DNA (the *Schistosoma* ITS and *S. mansoni* ND5 loci were detected and amplified using 0.02 ng template DNA) and *S. rodhaini* DNA (the *Schistosoma* ITS locus was detected and amplified using 0.2 ng template DNA). These were both deemed above that necessary to detect DNA from a single miracidium of both *S. mansoni* and *S. rodhaini* (~2.5 ng/μl and ~2.1 ng/μl, respectively, as measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massacuhsetts, USA) (Archer et al., unpublished data). Furthermore, during controlled laboratory infection of *B. glabrata* with *S. mansoni* miracidia testing, the *S. mansoni* ND5 locus was detected and visibly amplified (albeit, faintly) in four of all five *B. glabrata* exposed to just one *S. mansoni* miracidium 24 h post miracidia exposure. The reason the *S. mansoni* ND5 locus failed to amplify in one of these *B. glabrata* samples exposed to one single miracidium is likely because the miracidium failed to penetrate snail soft tissues but was then missed by microscopy following exposure; however, it is not possible to confirm this. Additionally, it should also be noted that in all five of these samples, the *Schistosoma* ITS locus failed to visibly amplify. The *S. mansoni* ND5 locus was also detected and visibly amplified in all five *B. glabrata* exposed to five *S. mansoni* miracidium 24 h post miracidia exposure. All five of these amplicons were more visible than those produced by *B. glabrata* infection with just one *S. mansoni* miracidium. Similarly, however, the *Schistosoma* ITS locus failed to visibly amplify in two of these five samples.

This is likely caused by two primary factors. First, the ETTS2 forward and ETTS1 reverse primers appear to have lower sensitivity in detecting and amplifying the *Schistosoma* ITS locus when compared to the sensitivity of the *S. mansoni* ND5 primer set in detecting and amplifying the *S. mansoni* ND5 locus. This was demonstrated during analytical sensitivity testing using serially diluted *S. mansoni* and *S. rodhaini* adult worm DNA and is, itself, likely caused by the significantly lower copy-number per cell of nuclear DNA when compared to mitochondrial DNA (whilst the *Schistosoma* nuclear ITS region is tandemly repeated many times, multiple thousand copies of mitochondrial DNA will be present within a cell), as well as by PCR biases for smaller amplicons at reduced DNA concentrations. Secondly, this is likely also caused by ETTS2 forward and ETTS1 reverse primers being depleted during amplification of the *Biomphalaria* ITS region as a much greater concentration of *Biomphalaria* DNA will be present within PCR reactions. A similar outcome was found previously when using the same ETTS2 forward and ETTS1 reverse primers to detect trematode infections within *Bulinus* spp. freshwater snail hosts (Pennance et al., 2020). It should therefore be noted that very early pre-patent (< 24 h post-exposure) *Biomphalaria* spp. infections with only few non-*S. mansoni* trematode miracidia may not be detected using this assay.

During assay pilot application, all 92 *Biomphalaria* DNA isolates collected from a *S. mansoni*-endemic area successfully detected and amplified the *Biomphalaria* ITS locus. As such, no reactions were considered to have failed either DNA extraction or PCR and the high-throughput BioSprint 96 DNA extraction protocol was deemed reliable as a means of isolating DNA from freshwater snail tissues, as demonstrated previously (Pennance et al., 2020). The assay also successfully detected the *S. mansoni* infection in *Bg*+*Sm*(mal) sample DNA as well as, importantly, two *Biomph*91(mal) DNA samples not found to be infected during cercarial shedding. Interestingly, one of these *S. mansoni*-infected *Biomph*91(mal) DNA samples (Fig. 4, sample B), only faintly amplified the *Schistosoma* ITS locus, suggesting that this may be a recently established infection with very few, or just one, *S. mansoni* miracidia. In addition, the assay successfully detected non-*S. mansoni* trematode infections in two *Biomph*91(mal) DNA samples, later confirmed as *Uvulifer* spp. Whilst there appears to be no available studies investigating whether established *Uvulifer* spp. infections within *Biomphalaria* spp. freshwater snails impact *S. mansoni* development and transmission, other trematode species have been found to influence *S. mansoni* development within *Biomphalaria* spp. (Laidemitt et al., 2019), and so this may also be the case for *Uvulifer* spp. infections. Nevertheless, this finding is of both ecological and zoological interest, and, to our understanding, is the first report of *Uvulifer* spp. infecting *Biomphalaria* in this area.

The current study, and molecular xenomonitoring more broadly, does have some limitations. Whilst end-point PCR is relatively inexpensive compared to more sophisticated PCR approaches such as real-time or quantitative PCR (qPCR), this molecular xenomonitoring assay does still require costly reagents and laboratory equipment, as well as specialised personnel. In addition, the assay requires sophisticated laboratory infrastructure seldom available in schistosomiasis-endemic areas. As such, the continued development of more portable, rapid, and easy-to-use nucleic acid amplification technologies that can be carried out at the point-of-need, such as LAMP and RPA/RAA, for molecular xenomonitoring purposes is encouraged here (Mesquita et al., 2022). For example, the development and validation of a duplex RPA/RAA assay capable of detecting both extremely low levels of *S. mansoni* DNA as well as an internal *Biomphalaria* control locus. Furthermore, the continued development of DNA extraction technologies capable of isolating DNA from freshwater snail tissues in resource-poor settings is also encouraged (Mesquita et al., 2022).

Whilst the assay proved to be high throughput, pooling multiple collected *Biomphalaria* specimens (for example, those collected from one individual malacological collection site) to be screened within one PCR reaction may further dramatically increase assay throughput whilst still allowing for the identification of transmission sites, as suggested previously (Pennance et al., 2020). This would, however, require an extremely sensitive molecular xenomonitoring protocol capable of detecting *S. mansoni* DNA within a much greater concentration of *Biomphalaria* DNA, for example, potentially real-time PCR. Nevertheless, pooling strategies such as this should be explored.

Additionally, the development and application of species-specific *Biomphalaria* internal reaction control loci would be of great malacological interest, allowing for the rapid identification of morphologically similar *Biomphalaria* species that may differ in their ability to transmit *S. mansoni* (e.g. *B. pfeifferi* and *B. sudanica*; Lu et al., 2016; Mutuku et al., 2017), without the need to generate sequence data (Pennance et al., unpublished data). Using species-specific *Biomphalaria* loci would also mean that any morphologically similar non-*Biomphalaria* genus of freshwater snails that do not transmit *S. mansoni*, such as *Gyraulus* spp., could also be identified and omitted from any analysis. Similarly, whilst *S. mansoni* sister-species *S. rodhaini* is not known to infect humans, rare occurrences of natural hybridisations between *S. mansoni* and *S. rodhaini* have been identified in Kenya and Tanzania (Leger and Webster, 2017) and so the detection and monitoring of *S. rodhaini* transmission within *Biomphalaria* is not only of zoological interest, but also of potential medical importance. As such, the implementation of an additional assay locus, capable of detecting *S. rodhaini* whilst also differentiating *S. rodhaini* from *S. mansoni* within *Biomphalaria* hosts, is encouraged. Interestingly, the *S. mansoni* ND5 primer set used here has previously been shown to detect and amplify an ~800-bp region of the *S. rodhaini* ND5 gene (Rwanda strain), and so clarification on the specificity of this DNA target is needed (Lu et al., 2016).

5. Conclusions

Here, we developed and validated a high throughput, easily interpreted, and reliable molecular xenomonitoring assay for the detection of *S. mansoni* and other trematode species within *Biomphalaria* spp. freshwater snail hosts. Furthermore, we applied this assay to successfully detect *S. mansoni* and non-*S. mansoni* trematode infections within *Biomphalaria* freshwater snails collected from a *S. mansoni*-endemic area. Molecular xenomonitoring assays such as this can be used to identify highly focal *S. mansoni* transmission sites, even in areas of low endemicity. Their continued development and use, particularly in areas nearing disease elimination, has been recommended by the WHO and will aid in improving disease control efforts, significantly reducing disease-related morbidities experienced by those in schistosomiasis-endemic areas.

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Ethical approval

Animal use was approved by the Institutional Animal Care and Use Committee (IACUC) of the Biomedical Research Institute for the Animal Use Protocol, #18-01. Ethical approval and research authorisations for the collection of *Biomphalaria* spp. from the southern shoreline of Lake Malawi, Malawi, were approved in the UK by the Liverpool School of Tropical Medicine (LSTM) Research Ethics Committee (application 17-018) and in Malawi by the National Health Sciences Research Committee (1805).

CRediT authorship contribution statement

John Archer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Shi Min Yeo:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Project administration. **Grace Gadd:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Project administration. **Tom Pennance:** Methodology, Writing – review & editing. **Lucas J. Cunningham:** Resources, Writing – review & editing. **Alexandra Juhàsz:** Resources, Writing – review & editing. **Sam Jones:** Resources, Writing – review & editing. **Priscilla Chammudzi:** Resources, Writing – review & editing. **Donales R. Kapira:** Resources, Writing – review & editing. **David Lally:** Resources, Writing – review & editing. **Gladys Namacha:** Resources, Writing – review & editing. **Bright Mainga:** Resources, Writing – review & editing. **Peter Makaula:** Resources, Writing – review & editing, Project administration. **James E. LaCourse:** Resources, Writing – review & editing, Project administration. **Sekeleghe A. Kayuni:** Resources, Writing – review & editing, Project administration. **Janelisa Musaya:** Resources, Writing – review & editing, Project administration, Funding acquisition. **J. Russell Stothard:** Resources, Writing – review & editing, Project administration, Funding acquisition. **Bonnie L. Webster:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the conclusions of this article are included within the article.

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